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Hybrid Hepatitis B Virus Core–Pre-S Proteins Synthesized in Avirulent *Salmonella typhimurium* and *Salmonella typhi* for Oral Vaccination

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Avirulent salmonellae expressing foreign genes are attractive for use as oral vaccine carriers. To facilitate the stable expression of heterologous genes without conferring antibiotic resistance, a deletion of the *asdA1* gene was introduced into *Salmonella typhimurium* and *S. typhi* Δ*cya* Δ*crp* mutant vaccine strains. An *asd*-complementing plasmid expressing hybrid hepatitis B virus nucleocapsid–pre-S (HBcAg–pre-S) particles was constructed. These hybrid HBcAg–pre-S particle genes were stably expressed in *S. typhimurium* and *S. typhi* Δ*cya* Δ*crp* mutant vaccine strains in this balanced, lethal host-vector combination. A single oral immunization of BALB/c mice with a recombinant *S. typhimurium* Δ*cya* Δ*crp* mutant synthesizing hybrid HBcAg–pre-S elicited potentially virus-neutralizing anti-pre-S serum immunoglobulin G antibodies. In addition, serum immunoglobulin G recognizing *S. typhimurium* lipopolysaccharide was induced. Distribution in tissue after oral immunization was analyzed in one plasmid-strain combination. The recombinant *S. typhimurium* colonized the gut-associated lymphoid tissue and the spleen and persisted for over 4 weeks, retaining the HBcAg–pre-S expression plasmid. An isogenic virulence plasmid-cured *S. typhimurium* Δ*cya* Δ*crp* strain expressing the same HBcAg–pre-S gene had reduced immunogenicity for the carried antigen after oral immunization.

Salmonella species causing typhoid fever in their natural hosts after infection via the oral route are invasive across the gut mucosa and colonize gut-associated lymphoid tissues, as well as the spleen and liver. Deletion of various genes can render *Salmonella* species avirulent while preserving different degrees of invasiveness (for reviews, see references 3 and 22). Such avirulent recombinant *Salmonella* strains are not only potentially useful as oral typhoid vaccines but also interesting candidates as oral vaccine carriers for heterologous (bacterial, viral, and parasitic) antigens. Many eukaryotic proteins and viral membrane and outer capsid proteins, which are preferential targets for induction of virus-neutralizing immunity, cannot be synthesized in their native conformation in prokaryotes. Epitopes defined as synthetic peptides, however, are generally insufficiently immunogenic by themselves and require a carrier moiety. Also, only a minority of bacterial proteins elicits high-titer serum antibody responses after oral immunization, at least in mice, with recombinant avirulent *Salmonella typhimurium* (23). To construct multivalent oral antiviral vaccines based on the synthesis of viral epitopes in live attenuated *Salmonella* spp., suitable carrier proteins may be required to present the desired viral epitopes in an immunogenic form to the host. Because introduction of additional antibiotic resistance markers is not desirable in a live vaccine strain, expression strategies have to be developed which use chromosomal integration of expression cassettes or plasmids without antibiotic resistance markers.

Infection with hepatitis B virus (HBV) continues to be a major public health problem, with over 200 million chronic carriers worldwide (for reviews, see references 13 and 29). Despite the existence of safe and efficient plasma-derived or recombinant vaccines based on the small envelope antigen (HBsAg) with or without parts of the middle (pre-S2) and large (pre-S1) envelope proteins, these vaccines are not available for the mass vaccination programs required to eliminate HBV. Oral vaccines, preferably in the form of multivalent single-dose vaccines, are desirable for such measures. Peptides representing B-cell epitopes of the HBV pre-S proteins inducing protective immunity have been well characterized (9, 13, 14, 19, 33). The viral nucleocapsid antigen (HBcAg), an internal antigen, also elicits protective immune responses (10, 16, 17, 27), possibly by providing T-cell help for anti-surface antibody production (15, 27), and has been defined as a carrier moiety enhancing the immunogenicity of fused heterologous B-cell epitopes (26). Hybrid HBcAg genes can be stably expressed in prokaryotes. The translation products self-assemble to form 27-nm-diameter particles (26) and are highly immunogenic when administered in live recombinant *S. typhimurium* and *S. dublin* by the oral route (24, 25, 28). In this report, we describe the construction of vectors for the expression of such hybrid HBc–pre-S genes without antibiotic resistance markers in avirulent Δ*cya* Δ*crp* Δ*asd* mutant *S. typhimurium* and *S. typhi* and the partial immunological characterization of the *S. typhimurium* carrier strains in the mouse model.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The strains were grown in LB medium (tryptone, 10 g/liter; $NACl_2$, 5 g/liter; yeast extract, 5 g/liter; glucose, 0.5 g/liter) (Difco, Sigma) supplemented with diaminopimelic acid (DAP) (50 μg/ml) when Δ*asd* mutant strains without an *asd*-complementing plasmid were grown and with

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
E. coli χ6097	F⁻ Δ(*lac-pro*) *rpsL* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi* φ80δ*lacZ*ΔM15	18
S. typhimurium		
χ4064	SR-11 pStSR101⁺ *gyrA1816* Δ*crp-1* Δ*cya-1*	4
χ4072	SR-11 pStSR100⁻ *gyrA1816* Δ*crp-1* Δ*asdA-1* Δ(*zhf-4*::Tn*10*) Δ*cya-1*	18
χ4217	SR-11 pStSR101⁺ *gyrA1816* Δ*crp-1* Δ*asdA-1* Δ(*zhf-4*::Tn*10*) Δ*cya-1*	This study
χ4550	SR-11 pStSR101⁺ *gyrA1816* Δ*crp-1* Δ*asdA-1* Δ(*zhf-4*::Tn*10*) Δ*cya-1*	This study; Asd⁻ derivative of χ4064
S. typhi		
χ4297	Ty2 Δ*crp-11* Δ(*zhc-1431*::Tn*10*) Δ*asdA1* Δ(*zhf-4*::Tn*10*) Δ*cya-12* Δ(*zid-62*::Tn*10*)	This study; Asd⁻ derivative of χ3927; 11
χ4417	ISP1820 Δ*crp-10* Δ(*zhc-1431*::Tn*10*) Δ*asdA1* Δ(*zhf-4*::Tn*10*) Δ*cya-12* Δ(*zid-62*::Tn*10*)	This study; Asd⁻ derivative of χ4346; 11
χ4435	ISP1820 Δ*crp-11* Δ(*zhc-1431*::Tn*10*) Δ*asdA1* Δ(*zhf-4*::Tn*10*) Δ*cya-12* Δ(*zid-62*::Tn*10*)	This study; Asd⁻ derivative of χ4323; 11

ampicillin (100 μg/ml) when χ4064(pNS27-53PS2) was grown. For Western blotting (immunoblotting) or immunization of animals, the bacteria were grown at 37°C with continuous shaking overnight. For the immunization experiments (see Tables 2 and 3), bacteria from static overnight cultures were used, and for the other experiments (see Tables 4 and 5 and Fig. 4), overnight cultures were diluted 1/20 in prewarmed LB and grown under aeration at 37°C to an optical density at 600 nm (OD_{600}) of 0.8 to 0.9 (~10^9 CFU). CFU were determined by plating serial dilutions on LB agar plates or MacConkey agar supplemented with 1% maltose. *Salmonella* spp. were transformed by electroporation as previously described (24).

Construction of *S. typhimurium* and *S. typhi* strains with a Δ*asdA1* mutation. Wild-type, virulent *S. typhi* Ty2 (type E1), *S. typhi* ISP1820 (type 46), and *S. typhimurium* SR-11 were genetically modified by using classic genetic methods similar to those previously described (4, 18). Stable maintenance and high-level expression of cloned genes on recombinant plasmids in avirulent *Salmonella* strains are dependent upon use of a balanced, lethal host-vector system. For this, a chromosomal mutation of the *asd* gene encoding aspartate β-semialdehyde dehydrogenase is introduced into Δ*cya* Δ*crp* mutants to impose an obligate requirement for DAP, which is an essential constituent of the rigid layer of the bacterial cell wall and is not synthesized in animals. The chromosomal Δ*asd* mutation is then complemented by a plasmid cloning vector possessing the wild-type *asd*⁺ gene. Loss of the plasmid results in DAP-less death and cell lysis.

The construction consisted of mobilization of the Δ*asdA1* mutation that had been isolated and characterized in *S. typhimurium* LT2-Z (18) into the Δ*cya* Δ*crp* mutant *S. typhi* and *S. typhimurium* strains. This was accomplished by placing silent transposon Tn*10* (*zhf-4*::Tn*10* encoding tetracycline resistance) near the Δ*asdA1* mutation and transducing the linked traits into *S. typhi* Ty2 Δ*cya-12* Δ*crp-11* mutant χ3927, ISP1820 Δ*cya-12* Δ*crp-11* mutant χ4323, and ISP1820 Δ*cya-12* Δ*crp-10* mutant χ4346 and *S. typhimurium* Δ*cya-12* Δ*crp-11* mutant χ4064 via P22HT*int* transduction with selection for tetracycline resistance and subsequent screening for the DAP-negative phenotype. Fusaric acid-resistant, tetracycline-sensitive derivatives of the Asd⁻ tetracycline-resistant *S. typhi* and *S. typhimurium* cotransductants described above were selected and screened by the method of Maloy and Nunn (12). *S. typhi* Δ*cya-12* Δ*asdA1* Δ*crp-11* mutant χ4297 and χ4335 and *S. typhi* Δ*cya-12* Δ*asdA1* Δ*crp-10* mutant χ4417 were then characterized for the presence of complete lipopolysaccharide (LPS), Vi antigen, flagellae, and auxotrophy for cysteine, tryptophan, arginine (for the Δ*crp-10* mutation), methionine, threonine, and DAP on minimal media supplemented with 0.5% glucose. *S. typhimurium* χ4550 was characterized for the presence of complete LPS, and flagellae and growth on minimal media supplemented with 0.5% glucose.

S. typhimurium χ4217 was derived by conjugating χ4072 (18) with *S. typhimurium* LT-2Z RecA⁻ Hsd⁻ containing R1drd19 (Cm^r) and pStSR101 (Tc^r) (8); screening for a tetracycline-resistant, chloramphenicol-sensitive isolate; and confirming the presence of virulence plasmid pStSR101 by agarose gel electrophoresis.

Vectors. Vector pNS27-53PS2, coding for a hybrid HBc–pre-S1–pre-S2 antigen (HBcAg amino acids 1 to 75, pre-S1 amino acids 27 to 53, HBcAg amino acids 81 to 156, and pre-S2 amino acids 133 to 143), has been previously described (25, 26). In this vector, the hybrid HBcAg–pre-S gene is expressed under control of a *tac* promoter and the vector has a ColE1 origin of replication. Vector pYAN was constructed from vector pYA292 (7; carrying a p15A origin of replication) by oligonucleotide-directed PCR mutagenesis by using a recently reported protocol (25). In short, cesium chloride gradient-purified DNA of vector pYA292 was amplified by using oligonucleotides 5′-GGG CCA TGG GAA TTC GCA ATT CCC GGG GAT CCG-3′ and 5′-GGG CCA TGG TCT GTT TCC TGT GTG AAA TTG TTA TCC G-3′. The amplified DNA was digested with restriction enzyme *Nco*I (all enzymes were purchased from Boehringer Mannheim), ligated with T4 ligase, and transformed into χ6097 grown in the absence of DAP by using standard techniques. Bacterial clones were picked, and the plasmid DNA was purified by rapid alkaline plasmid lysis and screened for the presence of an *Nco*I site. One vector containing an *Nco*I site and complementing Δ*asd* in χ6097 was further characterized by dideoxy sequencing of the polylinker. A map of the polylinker and its translated sequence in pYAN is displayed in Fig. 1. pYAN allows "authentic" expression of heterologous genes by replacement of the AUG within the *Nco*I site. The hybrid core–pre-S gene fragment of vector pNS27-53PS2 was amplified by PCR with simultaneous

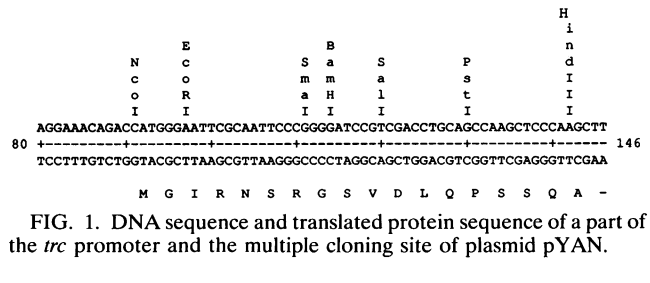

FIG. 1. DNA sequence and translated protein sequence of a part of the *trc* promoter and the multiple cloning site of plasmid pYAN.

introduction of an *Nco*I site at the HBcAg gene ATG by using the primer pair 5′-GGG GGA TCC ATG GAC ATC GAC CCT TAT AAA G-3′ and 5′-ATC CGT CAG GAT GGC CTT C-3′, digested with *Nco*I and *Hin*dIII, and ligated into pYAN digested with *Nco*I-*Hin*dIII. The ligation mixture was transformed into χ6097 and plated in the absence of DAP. Clones expressing the hybrid core genes were identified by immunoscreening with HBV pre-S2-specific monoclonal antibody 5520, and the DNA sequence of the entire insert in one of the resulting immunoscreen-positive vectors was verified by dideoxy sequencing with T7 polymerase (U.S. Biochemicals).

Immunoblotting and immunoscreening. For immunoblotting, cells from overnight cultures were taken up in 2× sample buffer and boiled for 10 min. Proteins were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). The proteins were subsequently transferred to nitrocellulose; incubated with monoclonal antibodies specific for HBV pre-S1 (18/7; courtesy of Wolfram Gerlich) (6), pre-S2 (5520; courtesy of Makoto Mayumi) (20), and denatured HBcAg (kindly provided by Vadim Bichko) (1); developed with peroxidase-coupled goat anti-mouse immunoglobulin G (IgG) (heavy and light chains; Medac); and visualized on X-ray film (Kodak) after incubation with a chemiluminescent substrate (ECL; Amersham). For immunoscreening, the following procedure was followed. Bacterial colonies were lifted onto nitrocellulose filters and lysed in 1% SDS for 30 min at 70°C. Free binding sites on nitrocellulose filters were blocked by 10% horse serum in Tris-HCl-buffered saline. Subsequently, immunoscreens were treated like immunoblots and secondary goat anti-mouse IgG (heavy and light chains; Medac) was visualized with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (Promega).

Immunizations. Groups of five female BALB/c mice (6 weeks old) were orally immunized by gavage with a single dose in 0.5 ml of phosphate-buffered saline containing 10^9 CFU of χ4064(pNS27-53PS2), χ4072(pYNS27-53PS2), or χ4217 (pYNS27-53PS2) or two doses 3 days apart (see Tables 2 and 3). For other experiments (see Tables 4 and 5 and Fig. 4), female 8-week-old BALB/c mice were deprived of water and food for 4 h and then fed 30 μl of 10% (wt/vol) sodium bicarbonate to neutralize stomach acidity (pipetted into the back of the mouth with a micropipettor). Thirty minutes later, the bacterial suspension (~1×10^9 to 2×10^9 CFU) was fed with a micropipettor in 20 μl of buffered saline with gelatin (2). Blood was taken by retroorbital puncture at biweekly intervals for 8 weeks after the first immunization. Serum was stored at −70°C prior to analysis.

Antibodies. Serum antibodies against HBV pre-S1 were determined with enzyme-linked immunosorbent assay (ELISA) techniques by using previously published protocols (26). A 200-ng sample of a synthetic peptide representing pre-S1 subtype *adw* amino acids 32 to 53 or a recombinant polypeptide representing the entire pre-S1 and pre-S2 sequences (5) was coated onto the microtiter plates in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Free binding sites on the plastic were blocked with a blocking buffer (BB) consisting of phosphate-buffered saline with 0.1% bovine serum albumin (Sigma) and 1% inactivated human AB serum (Sigma). Serial serum dilutions were incubated for 1 h at room temperature in BB, followed by goat anti-mouse IgG (Medac) which was detected by using peroxidase-coupled swine anti-goat Ig (Medac) and orthophenylenediamine as the substrate, which was read in an ELISA reader at 492 nm. To determine anti-LPS antibodies, a commercially available *S. typhimurium* LPS preparation (Sigma) was coated onto microtiter plates in carbonate buffer (pH 9.6) at 100 ng per well. Serum dilutions resulting in OD_{492} values three times those of preimmune sera are reported as titers.

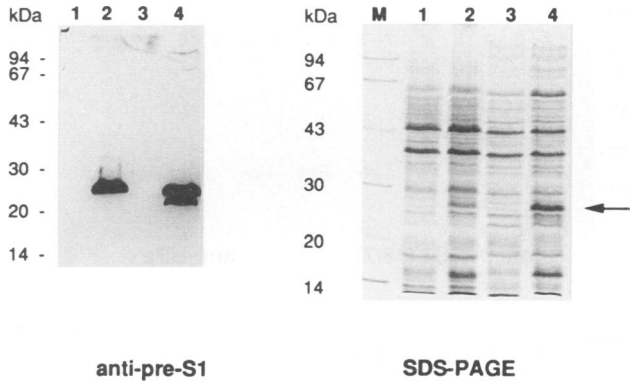

FIG. 2. Expression of hybrid HBcAg–pre-S protein off plasmid pNS27-53PS2 in χ4064 and plasmid pYNS27-53PS2 in χ4072. Proteins in whole bacterial cell lysates from overnight cultures of χ4072 (lane 1), χ4072(pYNS27-53PS2) (lane 2), χ4064 (lane 3), and χ4064(pNS27-53PS2) (lane 4) were separated by SDS–15% PAGE and stained with Coomassie brilliant blue (right panel) or transferred to nitrocellulose and visualized by immunostaining with a monoclonal anti-pre-S1 antibody (18/7; kindly provided by W. Gerlich) (left panel). The arrow indicates the position of the recombinant antigen in the Coomassie blue-stained gel.

Statistics. Statistical analysis was performed with the two-sided Wilcoxon test. $P \leq 0.05$ was regarded as significant.

RESULTS

Expression of an internally fused hybrid HBcAg–pre-S gene in *S. typhimurium* and *S. typhi*. The Δ*cya* Δ*crp* mutant *S. typhimurium* strain transformed with plasmid pNS27-53PS2 stably expressed the hybrid HBcAg–pre-S gene (Fig. 2). The HBcAg–pre-S hybrid displayed pre-S1 and pre-S2 antigenicity in immunoblots (Fig. 2 and 3) as previously demonstrated (25). The level of expression in an overnight culture was above 1% of the total cellular proteins, as estimated by Coomassie brilliant blue-stained SDS-PAGE. To express the same hybrid gene without antibiotic resistance markers, we first constructed an Asd^+-complementing expression vector (pYAN) with an ATG under *trc* promoter control that could be replaced by *Nco*I restriction (Materials and Methods and Fig. 1). The hybrid HBcAg–pre-S gene from vector pNS27-53PS2 was subcloned into pYAN as a PCR-amplified *Nco*I-*Hin*dIII fragment, resulting in vector pYNS27-53PS2. Expression of the hybrid HBcAg–pre-S gene was first verified by immunoblotting of *E. coli* χ6097 whole-cell extracts (data not shown). Subsequently, the vector was purified from χ6097 and transformed into the various Δ*cya* Δ*crp* Δ*asd* mutant *S. typhimurium* and *S. typhi* vaccine strains listed in Table 1. Pre-S1, pre-S2, and denatured HBc antigenicity of the hybrid HBcAg–pre-S protein from pYNS27-53PS2 in Δ*cya* Δ*crp* Δ*asd* mutant *S. typhi* and one *S. typhimurium* strain is shown in Fig. 3. In all of these strains, it was possible to express the hybrid HBcAg–pre-S gene stably to a high level from the Asd^+-complementing vector in the absence of DAP. The hybrid core–pre-S gene was expressed with this system without antibiotic resistance in avirulent *S. typhi* carrier strains suitable for phase 1 clinical testing (Fig. 3).

Oral immunogenicity of isogenic Δ*asd* Δ*cya* Δ*crp* mutant *S. typhimurium* vaccine with and without virulence plasmid strains expressing an HBcAg–pre-S hybrid. We next investi-

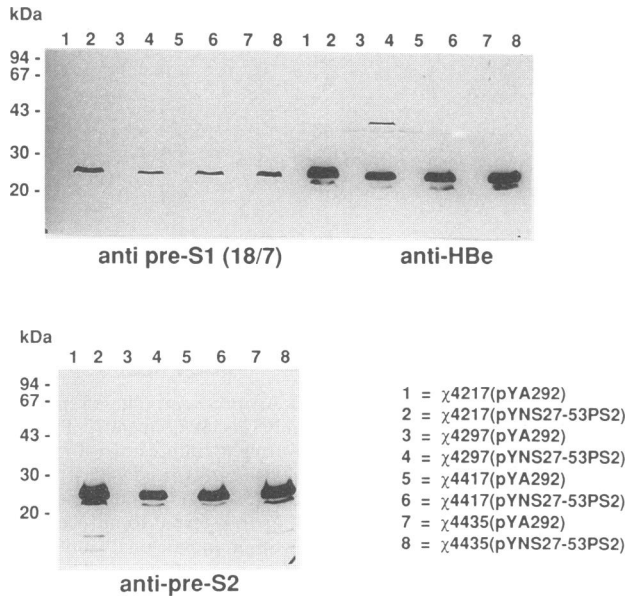

FIG. 3. Expression of hybrid HBcAg–pre-S protein off plasmid pYNS27-53PS2 in various *S. typhi* and *S. typhimurium* Δ*asd* Δ*cya* Δ*crp* mutant vaccine strains. Bacterial whole-cell lysate proteins were separated by SDS–15% PAGE, transferred to nitrocellulose, and visualized by immunostaining. The first antibody was a monoclonal antibody reactive with HBV pre-S1 (18/7), a monoclonal antibody recognizing denatured HBc/eAg (14E11; kindly provided by Vadim Bichko), or a monoclonal antibody directed against HBV pre-S2 (5520; kindly supplied by M. Mayumi), as indicated.

gated the oral immunogenicity of an internally fused hybrid HBcAg–pre-S gene expressed under *tac* promoter control from an ampicillin resistance plasmid (pNS27-53PS2) (ColE1 origin of replication) or under *trc* promoter control from an Asd^+-complementing vector (pYNS27-53PS2) (p15A origin of replication) in Δ*cya* Δ*crp* mutant *S. typhimurium* isogenic but the Δ*asd* and the presence of the virulence plasmid. When mice were orally immunized with strain χ4064(pNS27-53PS2), they developed pre-S1-specific serum IgG within 4 weeks after one oral immunization (Table 2). A second immunization within 3 days after the first immunization did not greatly enhance the anti-pre-S1 titers. The anti-pre-S1 titers 6 weeks after a single immunization were not significantly different from those 6 weeks after two immunizations 3 days apart with any of the strain-plasmid combinations used for immunization (Table 3; two-sided Wilcoxon test).

This is the first report of the induction of high-titer antibody responses against a heterologous epitope after a single oral immunization with live recombinant *S. typhimurium* strains. Immunization with χ4072(pYNS27-53PS2) or χ4217(pYNS27-53PS2), two *S. typhimurium* strains with p15A-based Asd^+ vectors carrying the hybrid HBc genes, also led to serum anti-pre-S1 antibodies, but the titers achieved (Table 2) and the rate of seroconversion (Table 3) appeared somewhat lower than those obtained with χ4064(pNS27-53PS2). It should be noted that ColE1-based plasmid pNS27-53PS2 reached a higher copy number per cell and, in consequence, a higher level of expression than p15A-based plasmid pYNS27-53PS2 (compare Fig. 2). When comparing anti-pre-S1 titers at 6 weeks after a single immunization or two consecutive immunizations, we found that the titers achieved and the rate of seroconversion were lowest in mice fed virulence plasmid-cured recombinant strain χ4072(pYNS27-53PS2) (Table 3). Individual anti-pre-S1 titers were heterogeneous (Table 3), corresponding to previous observations after oral immunizations with a C-terminal HBcAg–pre-S fusion expressed in *aroA* mutant strains of *S. typhimurium* and *S. dublin* (24). The anti-pre-S1 titers against the internally fused sequence were higher than those previously observed against a C-terminally fused pre-S2 sequence, confirming results obtained after immunization of mice with purified particles (26).

When the protein pattern was analyzed by Coomassie blue-stained SDS-PAGE, the *asd cya crp* mutant strains used (χ4072 and χ4217) appeared to differ slightly, in addition to the Δ*asd* geno- and phenotype, from the *cya crp* mutant strain used in this comparison (χ4064) (unpublished observation). Therefore, an independent *asd* isogene of χ4064 was constructed and plasmid pYNS27-53PS2 was moved into the resulting new strain, χ4550(pYNS27-53PS2). In vitro levels of hybrid core–

TABLE 2. Pooled serum anti-pre-S1 IgG titers

Antibody and immunogen[a]	Dose (CFU)	Immunization day(s)	Reciprocal titer[b] at bleed time (wk) of:		
			2	4	6
Anti-32-53 *adw* synthetic peptide					
χ4072(pYNS27-53PS2)	10^9	1	0	200	800
χ4072(pYNS27-53PS2)	10^9	1, 3	0	0	1,600
χ4064(pNS27-53PS2)	10^9	1	0	1,600	3,200
χ4064(pNS27-53PS2)	10^9	1, 3	0	>12,800	>12,800
χ4217(pYNS27-53PS2)	10^9	1	0	3,200	3,200
χ4217(pYNS27-53PS2)	10^9	1, 3	0	3,200	3,200
Anti-pre-S *ayw* (complete pre-S1 and pre-S2)					
χ4072(pYNS27-53PS2)	10^9	1	0	0	200
χ4072(pYNS27-53PS2)	10^9	1, 3	0	0	0
χ4064(pNS27-53PS2)	10^9	1	0	1,600	1,600
χ4064((pNS27-53PS2)	10^9	1, 3	0	12,800	>12,800
χ4217(pYNS27-53PS2)	10^9	1	0	800	3,200
χ4217(pYNS27-53PS2)	10^9	1	0	200	800

[a] Female BALB/c mice were immunized orally by gavage with approximately 10^9 CFU of the indicated bacterial strains, either with a single dose or with two doses given 2 days apart.

[b] Each serum anti-pre-S IgG titer is the reciprocal of the highest serum pool ($n = 5$) dilution yielding an OD_{492} three times those of preimmune sera by a previously described ELISA technique (28) using a synthetic peptide representing subtype *adw* pre-S1 amino acids 32 to 53 or a recombinant globular pre-S protein (subtype *ayw*) (5) as the solid-phase reagent.

TABLE 3. Individual reciprocal titers of serum anti-pre-S1 IgG 6 weeks after immunization

Immunogen[a]	Dose (CFU)	Immunization day(s)	Sample	Reciprocal titer[b]
χ4072(pYNS27-53PS2)	10^9	1	1	800
			2	400
			3	400
			4	100
			5	100
χ4072(pYNS27-53PS2)	10^9	1, 3	6	0
			7	12,800
			8	0
			9	0
			10	0
χ4064(pNS27-53PS2)	10^9	1	1	3,200
			2	100
			3	6,400
			4	25,600
			5	6,400
χ4064(pNS27-53PS2)	10^9	1, 3	6	12,800
			7	800
			8	51,200
			9	100
			10	102,400
χ4217(pYNS27-53PS2)	10^9	1	1	1,600
			2	12,800
			3	3,200
			4	0
			5	3,200
χ4217(pYNS27-53PS2)	10^9	1, 3	6	0
			7	800
			8	12,800
			9	1,600
			10	100

[a] Female BALB/c mice were immunized orally by gavage with approximately 10^9 CFU of the indicated bacterial strains, either with a single dose or with two doses given 2 days apart.

[b] Each serum anti-pre-S IgG titer is the reciprocal of the highest dilution yielding an OD_{492} three times those of preimmune sera in an ELISA using a synthetic pre-S1 peptide (subtype *adw*, amino acids 32 to 53) as the solid-phase reagent.

pre-S protein synthesis determined by SDS-PAGE and Western blotting were identical to those observed in χ4072 and χ4217 (data not shown). When BALB/c mice were immunized with χ4550(pYNS27-53PS2), they produced anti-pre-S1 antibody titers comparable to those found after immunization with χ4217(pYNS27-53PS2) (Table 4). This demonstrates in a second independent experiment that reasonable antibody titers directed to an insert in a hybrid core antigen could be achieved after a single oral immunization when the hybrid gene was expressed off a non-antibiotic resistance-conferring vector. At the same time, the immunogenicity of the carrier strain for its own antigens was preserved, as exemplified by the induction of serum IgG anti-LPS in mice fed either the HBV recombinant strain or a vector-only control (Table 5). Consistent with the slightly reduced in vivo antigenic load (see below), the anti-LPS titers appeared to be higher in animals immunized with the vector control strain.

Persistence and tissue distribution of χ4550(pYNS27-53PS2) after oral immunization. Immunogenicity of a carried antigen is a complex feature related, among other factors, to the invasiveness of the carrier strain, its time of replication and

TABLE 4. Individual reciprocal titers of serum anti-pre-S1 IgG in BALB/c mice fed χ4550(pYNS27-53PS2)

Immunogen[a]	Sample(s)	Reciprocal titer[b]
χ4550(pYNS27-53PS2)	11[c]	3,200
	12	100
	13	800
	14	0
	15	3,200
	16	100
	17	1,600
	18	6,400
	19	51,200
	20	800
χ4550(pYAN)	1–10[d]	0

[a] Female BALB/c mice were fasted for 4 h, fed 30 μl of 10% sodium bicarbonate, and subsequently immunized orally with 2.2×10^9 CFU of χ4550 (pYAN) or 1.3×10^9 CFU of χ4550(pYNS27-53PS2) in 20 μl of BSG (2).

[b] Each serum anti-pre-S IgG titer is the reciprocal of the highest serum dilution yielding an OD_{492} three times those of preimmune sera in an ELISA using a recombinant globular pre-S protein as the solid-phase reagent.

[c] Individual sera 38 days after a single oral immunization.

[d] Pooled sera (negative control) 38 days after a single oral immunization.

persistence in host tissues, and the stability of the replicon, as well as the levels of expression achieved in vivo. As a first step towards analysis of some of these complex interactions, we looked at the in vivo tissue distribution of one of the recombinant *S. typhimurium* strains expressing a hybrid HBcAg–pre-S gene. After a single oral dose of χ4550(pYNS27-53PS2) or χ4550(pYAN), the vector-only control, the recombinant *S. typhimurium* reached the gut-associated lymphoid tissue and the spleen and persisted, with slowly declining titers for over 1 month (Fig. 4). In the 1 to 2 weeks after oral immunization, a low level of recombinant bacteria was detected in the blood and in lung tissue, emphasizing the systemic spread of the vaccine strain. Approximately concurrent with the elimination of bacteria from the circulation, they reached their highest titers in splenic tissues around day 15. The strain expressing the HBcAg–pre-S hybrid gene reached lower CFU titers in the spleen and Peyer's patches than did the Asd^+ plasmid control and was eliminated somewhat faster, consistent with a slight additional in vivo attenuation caused by overexpression of the carried HBcAg–pre-S gene. However, these indicate that expression of this heterologous gene compared with the vector-only control does not grossly disturb the in vivo growth characteristics of the carrier strain.

TABLE 5. Serum anti-LPS IgG titers in BALB/c mice fed χ4550(pYAN) or χ4550(pYNS27-53PS2)

Immunogen[a]	Reciprocal anti-LPS IgG titer[b] on day:			
	0	21	38	56
χ4550(pYAN)	0	100	6,400	12,800
χ4550(pYNS27-53PS2)	0	0	800	1,600

[a] Female BALB/c mice were fasted for 4 h, fed 30 μl of 10% sodium bicarbonate, and subsequently immunized orally with 2.2×10^9 CFU of χ4550 (pYAN) or 1.3×10^9 CFU of χ4550(pYNS27-53PS2) in 20 μl of BSG (2).

[b] Each serum anti-LPS IgG titer is the reciprocal of the highest serum pool ($n = 10$) dilution yielding an OD_{492} three times those of preimmune sera in an ELISA using *S. typhimurium* LPS (100 ng per well; Sigma) as the solid-phase reagent.

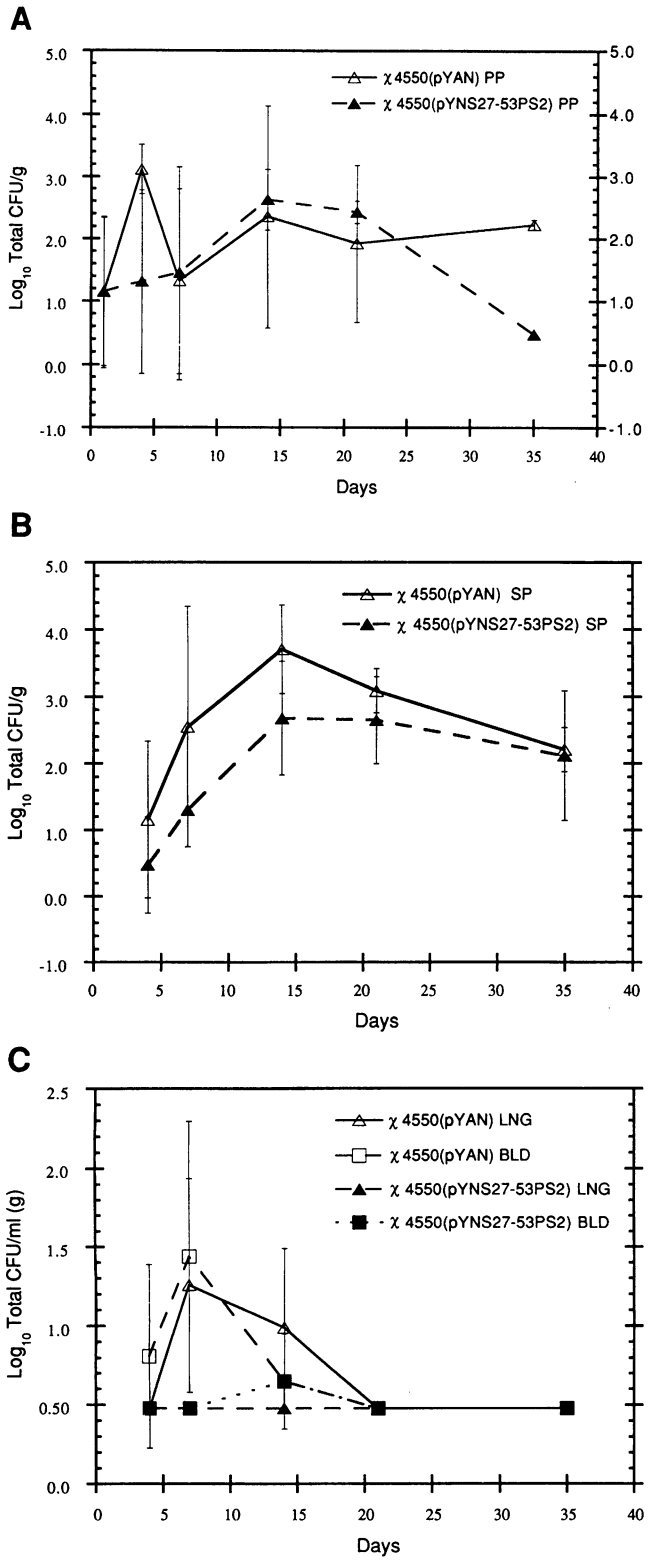

FIG. 4. Recovery of *S. typhimurium* Δ*cya-12* Δ*crp-11* Δ*asdA1* mutant χ4550(pYAN) and χ4550(pYNS27-53PS2) from Peyer's patches (PP) (A), spleens (SP) (B), and heart blood (BLD) and lungs (LNG) (C) of BALB/c mice at specified times after peroral inoculation with 2.2×10^9 CFU of χ4550(pYAN) and 1.3×10^9 CFU of χ4550 (pYNS27-53PS2). Three mice were sacrificed for each time point. The results are given as geometric means ± standard deviations.

DISCUSSION

The ultimate goal of the experiments presented here was the development of oral vaccines against HBV infection. Avirulent but invasive *Salmonella* spp. are attractive carriers of heterologous antigens that can be used to elicit mucosal and systemic immune responses by the oral route (3, 22). HBsAg, the major surface antigen of HBV, which is the primary component of current serum-derived or recombinant HBV vaccines, cannot be synthesized in an immunologically meaningful form in prokaryotes. HBsAg is a particulate eukaryotic antigen that is secreted from eukaryotic cells and in the process incorporates a unit membrane lipid bilayer. The critical epitopes on HBsAg are dependent on its conformation, and short of mimicking complex conformation-dependent epitopes and/or the secretion process and assembly of HBsAg particles in prokaryotes, it is not possible to use it for prokaryote-based vaccines. Alternative protective peptidic epitopes have been mapped on the pre-S1 and pre-S2 proteins of HBV (9, 13, 14, 19, 33), and HBcAg itself is a protective antigen (10, 16, 17, 27), although the mechanism of protection is not completely defined. We have previously constructed particulate hybrid HBV core–pre-S proteins by genetic engineering with *E. coli* that are capable of eliciting high-titer anti-pre-S1 antibodies (25, 26). These hybrid HBcAg–pre-S genes can be expressed to high levels in *E. coli*, *Salmonella* spp., and other prokaryotes, such as *Vibrio cholerae* and in *Yersinia enterocolitica* (23–26, 28, 30–32). For the testing and, ultimately, the use of *Salmonella* spp. as oral-route carriers of such hybrid antigens in humans, suitable non-antibiotic-resistant and stable expression systems have to be developed. A system which relies on in *trans* complementation of an *asd* gene deletion in the carrier strain genome by a copy of the *asd* gene on plasmids expressing heterologous genes has recently been described (7, 18). We first constructed a derivative (pYAN) of these vectors carrying the *S. typhimurium asd* gene by introducing a unique *Nco*I restriction site that permits replacement of the polylinker AUG with the AUG of the gene of interest to create an unfused heterologous gene copy for "authentic" expression. A previously described HBcAg–pre-S1–pre-S2 gene (25, 26), in which the region coding for the immunodominant antibody recognition site of HBcAg is replaced by a sequence coding for pre-S1 epitopes and two overlapping pre-S2 epitopes are fused to the 3′ end of the truncated HBc gene, was cloned into this low-copy-number p15A derivative. The HBcAg–pre-S hybrid gene was expressed to high levels in Δ*asd* Δ*cya* Δ*crp* mutant *S. typhimurium* strains in vitro. However, the levels of in vitro HBcAg–pre-S expression off this low-copy-number plasmid controlled by the *trc* promoter were below the levels observed from the original β-lactamase pBR322-based plasmid, pNS27-53PS2. When BALB/c mice were immunized with χ4064(pNS27-53PS2), they all seroconverted to relatively high serum anti-pre-S1 titers. This is the only epitope presentation system based on *Salmonella* vaccine strains that is capable of eliciting a high-titer serum antibody response after a single oral immunization. This is probably due to the high inherent immunogenicity of the HBV core antigen and the enhanced expression level of the recombinant protein. BALB/c mice immunized with Δ*cya* Δ*crp* Δ*asd* mutant *S. typhimurium* χ4217(pYNS27-53PS2) also seroconverted to anti-pre-S1 but at a lower rate and to lower titers than mice immunized with χ4064(pNS27-53PS2). The lower level of gene expression observed in vitro from the low-copy-number p15A-based plasmid than from the ColE1-based plasmid, with or without additional effects caused by the balanced, lethal Δ*asd* genotype, may account for the observed difference in immunogenicity. It was subsequently found that apart from

Δ*asd*, χ4217 also differed from χ4064 in the ability to colonize deep tissue of immunized mice and in the protein pattern observed on Coomassie blue-stained SDS-PAGE (unpublished observation). A virulence plasmid-cured derivative of χ4217, χ4072, was even further reduced in immunogenicity for the carried hybrid HBcAg–pre-S antigen. Absence of the virulence plasmid reduces transepithelial invasiveness of *S. typhimurium* after oral uptake (8, 21). Without formal proof, it is therefore reasonable to assume that the reduced immunogenicity is owed to a reduced antigenic load in transepithelial host tissues. It should also be noted here that it takes approximately 1 month to detect appreciable titers of serum IgG directed against the carried antigen after a single oral dose of recombinant *S. typhimurium*. Invasiveness and tissue persistence of the recombinant *S. typhimurium* carrier strain, as well as continued synthesis of the carried antigen in the host, probably contribute to its immunogenicity. Core antigen hybrids synthesized in prokaryotes are strictly cytoplasmic antigens. To be seen by host B cells, HBcAg–pre-S particles have to be released from the carrier bacteria. We therefore assume that lysis of the bacteria is a prerequisite for the observed immunogenicity of the carried antigen. χ4217(pYNS27-53PS2) differs from χ4064 (pNS27-53PS2) in the lower levels of HBcAg–pre-S hybrid gene expression than the original pBR322-based replicon, as well as by additional phenotypic differences that are unaccounted for. To pursue our major goal, to reproduce the comparatively high immunogenicity of the originally used pBR322-based replicon expressing the hybrid HBcAg–pre-S gene in an *S. typhimurium* Δ*cya* Δ*crp* strain (χ4064) in a more directly comparable strain with a non-antibiotic-resistant plasmid, we constructed a Δ*asd* isogene of this strain (χ4550). When tested in BALB/c mice, recombinant strain χ4550 (pYNS27-53PS2) colonized gut-associated lymphoid and splenic tissues somewhat less strongly but at a level comparable to that of a vector control, χ4550(pYAN), indicating that expression of the HBcAg–pre-S gene does not grossly interfere with the in vivo growth behavior of the carrier strain. The immunogenicity of χ4550(pYNS27-53PS2) for the carried antigen was similar to that observed with χ4217(pYNS27-53PS2) after oral immunization. We assumed that the lower level of expression off pYNS27-53PS2 than pNS27-53PS2 might account for the remaining difference in immunogenicity and constructed two different high-copy-number *asd*-complementing plasmids to address this question (34). It should be noted, however, that we lack adequate means to study in vivo expression of the heterologous antigens and that a difference in copy number of an *asd*-complementing vector brings additional variables, in addition to increased heterologous gene expression, that make it difficult to analyze conclusively the impact of gene dosage on immunogenicity in a carrier strain-vector combination. By using *asd*-complementing plasmid pYNS27-53PS2, it was possible to express the hybrid HBcAg–pre-S gene stably in Δ*cya* Δ*crp* Δ*asd* mutant *S. typhi* candidate vaccine strains, some of which have already been tested in phase 1 clinical trials with human volunteers. It will therefore be possible to test the immunogenicity of HBcAg–pre-S hybrids with human volunteers in the near future.

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